Mutagenesis manipulation of l-asparaginase

Abstract

Introduction: L-asparaginase (L-ASNase) is FDA approved enzyme which is widely used in pharmacy for treatment of acute lymphoblastic leukemia. Therefore, development of efficient procedure by rational mutagenesis manipulation of L-asparaginase to increase enzyme efficiency is in high interest.

Materials and Methods: Since several studies focused on key role of Asp90 in L-ASNase active site, we developed our stud to substitution of Asp90 through rational site directed mutagenesis method. Based on the crystal structure of ansB asparaginase II, bioinformatics analysis and docking binding energy measurement, D90A mutant was introduced by site-directed mutagenesis in order to suppress glutaminase activity but not asparaginase activity.

Results: Mutated construct was expressed in *E. coli* BL 21. The manipulated asparaginase was purified; their biochemical and kinetic investigation revealed thatrecombinant asparaginase WT and mutant have optimum temperature of 40 °C and pH 7. Result showed that mutation has no effect on the optimum pH and temperature of the enzyme while D90A catalytic efficiency values showed decreased of 21. 9 % for asparagine and 119% lower catalytic efficiency for glutamine substrates in compare with the WT asparaginase.

Conclusion: Findings provide a rationale for testing the hypothesis that a glutaminase-deficient asparaginase variant (D90A) will exhibit greater therapeutic index than that of WT against cancers and could be used in therapeutic drug development research.

Keywords: Asparaginase, *E. coli* , site-directed mutagenesis, molecular docking

Introduction:

One of the most widely used enzymes in the medical industry and food industry is the L-asparaginase enzyme [1]. The enzyme that divide the l-asparagine to aspartic acid and ammonia components. Normally, tumor cells are not able to produce L-Asparagine for the survival of the cell. Therefore, if these cells are treated with the L-asparaginase enzyme, they will be selectively eliminated because they are not able to replace the damaged L-asparagine amino acid without being harmed to healthy cells [2, 3].

The most common use of L- asparaginase is their use as a food processor [4, 5]. Asparaginases with the names Acrylaway and PreventASe are examples of this enzyme used in the process of food and reduces the amount of acrylamide produced as a carcinogen in foods with starch, including snacks and biscuits [6]. The L-asparaginase enzyme is an important factor in chemotherapy that is used for acute lymphoblastic leukemia (ALL) [7, 8]. The enzyme has been used as a major factor in the chemotherapy protocol for the treatment of children with ALL for almost 30 years [9]. Over the past 10 years, PEG L-asparaginase has been known as an alternative to L-asparaginase [10]. Elspar asparaginase is a drug for the treatment of acute lymphoblastic leukemia [11, 12]. This enzyme is also used in mastocytoma treatment protocols. Unlike most chemotherapy drugs, the enzyme can be given intramuscularly, subcutaneously or intravenously without tissue problems [13, 14]. Intravenous injection of L-asparaginase reduces

asparagine sources in the body and neoplastic cells are exposed to asparagine deficiency. Therefore, because of the inability to produce proteins, RNAs, and DNAs, they are stopped or even killed [15, 16].

The asparaginase treatment quality relies on the fact that the cancer cells of the blood and other tumor cells are not able to produce asparagine, while healthy cells can do this naturally. Cancer cells are dependent on asparagine in the cell for survival [17]. L-asparaginase derived from *Escherichia coli* has been used in the treatment of acute leukemia since 1960. This drug may be prescribed as an intravenous infusion or as an intramuscular injection. [18]. One of the most important solutions for the production of enzymes is industrial, using genetic engineering techniques for the production of proteins in the recombinant form [19, 20]. Since asparaginase has dual asparaginase and glutaminase activities with high affinity to glutamine which catalyzes the hydrolysis of glutamine to glutamate and NH3, this reaction will leads to decreases glutamine and increase NH3 in blood with toxic effect. As a result, reduction of blood glutamine which carries ammonia group caused severed issue in liver biochemical activity. Since several studies focused on key role of Asp90 in L-ASNase active site, we developed our study to substitution of Asp90 through rational site directed mutagenesis method. Knowledge of ansB asparaginase II EC: 3. 5. 1. 1crystal structure [21] and other similar asparaginase [22], beside on protein sequences studies of native ansB asparaginase II have enabled our rational design toward improving the enzyme efficiency. Importance role of aspartate amino acid in asparaginase enzymes active site caused 4 mutants (D90S, D90L, D90I, D90A) were selected in order to investigate its effect on L-ASNase

kinetic behavior. Since structural and docking predictions require experimental verification, we introduced D90A mutation with highest binding affinity to asparagine and lowest with glutamine substrates by using a site-directed mutagenesis method. During selection procedure, the binding energy of WT and mutants enzymes (4 candidate substitutions) with substrate calculated and compared with sole enzyme with no substrate as negative control.

Materials and Methods:

Strains, media and reagents

Escherichia coli DH5α was used for cloning and amplification of the gene and BL21 (DE3) as host for gene expression. pUC57 and pET-3a were used as cloning and expression vectors respectively. For growth of *E. coli* , the LB medium is also used. Preparation the Competent cells and Transfer of pUC57-ansB plasmid and Plasmid extraction and cutting with the enzyme were performed according to standard molecular Cloning methods [23]. *Taq* DNA polymerase, T4 DNA ligase and restriction enzymes were purchased from Thermo Fermentase (USA). Oligonucleotides were synthesized and automatic DNA sequencing was performed at Macrogene Inc (South Korea). Nessler's

reagent was obtained from Merck, Germany. L-asparagine, ammonium sulfate, ampicilline, kanamycine, IPTG and acryl amide were purchased from SIGMA, USA.

Rational design of mutations

Since glutaminase-deficient L-ASNase variant was the goal, we focused on reduction of glutaminase binding energy through decrease of glutamine affinity. In order to select the substitution point, the crystal structure of the ansB protein was used and docking study for affinity measurement through binding energy of WT and D90A mutant enzymes when docked with glutamine and aspargin ligand was carried out by Autodock 4. 0 to predict what mutated form(s) could be ideal for substitution to retain activity against asparagine but not glutamine. In order to investigate Asp90 substitution (D90S, D90L, D90I, D90A) in L-ASNase active site enzyme activity, the 3D structure of asparaginase (PDB 3ECA)is selected by ConText software. The structure of WT and mutated model asparaginases were designed by Pymol and Modeller software studies (Fig. 1). The WT and mutated structures were energy minimized by Chimera software.

Site-directed mutagenesis

Site-directed mutagenesis of ansB gene was conducted using QuikChange Site Directed Mutagenesis Kit (Stratagene, CA, USA). The pET-3a vector containing ansB gene (1049bp) with optimized codon preference for expression in *E. coli* was utilized to produce the mutant asparaginase. In this methods, double stranded, dam methylated pET-3a DNA with ansB gene isolated from *E. coli* DH5α was used with pair of designated complementary primers containing the desired point mutation (Table 1). The mutagenesis was extended by *pfu Turbo* DNA polymerase by using thermo cycling condition of 95°C for 3 min, 18 cycles at 95°C for 30s, 66°for 45s and 68°C for 1min/Kb of plasmid DNA length. The product was treated by *Dpn* I (Fermentase) at 37°C for 1 hour and nicked plasmid DNA was transformed to

E. coli BL21 by which the nick was repaired. The plasmids harboring desired mutation was identified by DNA sequence analysis [24, 25].

Asparaginase andglutaminaseactivity

Both wild type (WT) and mutated D90A were cultured in LB medium at 220 rpm and 37°C for 16-18 hours. After induction of transformed E. coli BL 21 hosts with IPTG, supernatant was collected by centrifuge. Expressed extracellular asparaginase was precipitated by ammonium sulfate at 12000 g and 4°C for 10 minutes and investigated by SDS-PAGE analysis[26].

Asparaginase activity was measured in an assay mixture at 37° C for 30 minutes and stop reaction by 0. 1 ml of TCA 1/5 mM. The mixture was centrifuged for 2 minutes at 13000 rpm and concentration of ammonia produced by 0. 5 ml of the Nessler's reagent was determined[27]. Additional the total protein was determined by Bradford assay and enzyme specific activity determined. Glutaminase enzyme activity was measured using a Glutamate Assay kit (Abcam) according to the manufacturer's instructions.

Purification and biochemical characterization of expressedasparaginase

Supernatant of WT and D90A asparaginase was collected through centrifuge at 13. 000 rpm for 15 min at 4°C. The filtered supernatants were concentrated by using Amicon centrifugal filter device (Cut-off, 30. 000). The concentrated supernatants were loaded on Superdex size Column for gel permeation chromatography (Amersham, Inc, USA), which has previously been equilibrated with 0. 1 M sodium acetate buffer (pH 7), then eluted with 0. 1M sodium acetate containing 0. 15M NaCl with flow rate of 2. 5ml min $^{-1}$

using AKTA Purifier Fast Protein Liquid Chromatography system (Amersham bioscience, USA). The fraction profiles of OD280 and asparaginase activity were analysed to determine the desired protein peaks. The peak fractions were pooled and concentrated by Amicon centrifugal filter device and stored at -20°C for further characterization[28].

The asparaginase and glutaminase activity assay was carried out using asparagine and glutamine at serial concentration and kinetic parameters, K_m and V_{max}, were determined. Each assay was performed in triplicate. The V_{max} values were converted to k_{cat} value, by normalizing the enzyme concentrations by the molecular mass of the monomer. The asparaginase pH profile was determined at 37°C with different reaction buffers pH values (2 to 9). The temperature profile of asparaginase was investigated in reaction buffer (pH 7) with 189 mM asparagine substrate at temperature ranging from 10 to 90°C with 10°C intervals for 30 minutes[27].

Statistical analysis

Statistical analyses were performed using Minitab (USA). The Student t test was used to compare mean differences. Significance was set at a $P value$ of < 0. 05.

Results

Asparaginaseexpression

The ansB gene which containing 1049 bp and open reading frame encoding 326 amino acid residue were designed by Gene Optimizer software and synthesized according to *E. coli* BL21 codon performance. The artificial ansB

gene was digested and ligated to pET-3a expression vector and pET-3a –
ansB recombinant plasmid construct prepared and transferred to *E. coli*
BL21 host. The presence of ansB gene in recombinant vector was confirmed
by specific primers. To promote expression of recombinant asparaginase, the
transformed host was induced by IPTG. Transformed colons supernatant
were collected and investigated by asparaginase activity assay and SDS-
PAGE in different sample dilution.

Experimental evidence indicated that in *E. coli* WT recombinant active
asparaginase expressed and successfully secreted extracellularly. The
expressed asparaginase revealed as a band on SDS-PAGE with molecular
size of 36 KDa(Fig. 2). The expressed WT and mutant asparaginase was
purified and characterized.

Construction of mutantasparaginase

The structure of WT and mutated ansB asparaginase II were designed and
docked against each other to determine the substrate binding affinity. The
docking results indicated that D90A substitution results in highest binding
energy and affinity to asparagine and lowest against glutamine ligand in
compare with other substitutions. Hence, single mutant of asparaginase was
developed by substituting D90A through site-directed mutagenesis. Having
transformed to *E. coli* BL 21, the mutated enzyme was purified and
characterized. The quantitative enzyme assay revealed that recombinant WT
and mutant asparaginase were expressed successfully with activity of 96. 05
± 0. 2 U/ml and 96. 09 ± 0. 6 U/ml with asparagine and glutamine substrates
respectively.

Effect of D90Amutation on pH and temperatureoptima

To determine pH range of activity and optimum pH of asparaginase, *E. coli* expressed WT and mutant recombinant asparaginase activity were determined at different pH. The optimum pH for both asparaginase functions is at pH 7 and virtually the mutation has no effect on the optimum pH of the enzyme.

In order to determine WT and mutant asparaginase behavior at different temperature, purified recombinant samples were assayed for asparaginase activity at wide range of temperatures. It was discovered that recombinant asparaginase WT and mutant have optimum temperature of 40 °C.

The results of the temperature profile showed that at lower temperatures might the molecular movement is less but with a gradual increase of temperature could cause higher molecular movements which lead to speed of the reaction and increasement of digested product. The result of pH and temperature profile is shown in Fig 3.

Characterization of engineeredasparaginasekinetic parameters

Kinetic analysis indicated that the k_m value of mutant for asparagine and glutamine substrates was 13. 5 and 48. 6 % respectively higher than the WT ($P < 0. 05$). Moreover, the catalytic efficiency values (k_{cat}/k_m) of the D90A mutant was decreased about 21. 9 % for asparagine and 119% lower catalytic efficiency for Glutamine substrates compared with the WT asparaginase (Fig. 4). In comparison of Asn/Gln *kcat* , between WT and mutant, results showed that *kcat* ratio of Asn/Gln in WT and mutant is 1. 45

and 2. 08 respectively, with higher enzyme turnover number for asparagine in mutant asparaginase. Furthermore, specificity constant (kcat/km) comparison of Asn/Gln ratio between WT and mutant calculated as 13. 35 and 24. 04 respectively, which shows relative efficiency for Asn versus Glm is higher for the mutant than for the WT.

Analysis of 3D structure

Modeling analysis and validity investigation of the WT and mutated models demonstrated that the three dimensional conformation of mutant model has the same structure as the WT enzyme (Fig. 1); therefore, it may be concluded that the structural domains were not affected by the substitution.

Discussion:

L-ASNase catalyzes the hydrolysis Asn and is widely used for the treatment of haematopoetic diseases such as ALL and lymphomas and therapeutic forms of asparaginase come from different biological sources (*E. coli* and *Erwinia chrysanthemi*) [21, 22]. The anti-neoplastic activity of this enzyme is due to its ability to reduce the storage of asparaginase in the body, due to the property of its asparaginase degradation. Cancer cells need more than usual amino acid asparagine and cannot produce it as they need it, because the level of asparagine synthesis is much lower than it needs, and to produce and survive Serum asparagine is dependent[29, 30].

Detailed understanding of the catalytic mechanism of asparaginase might permit the design of new forms of asparaginase with optimal biochemical properties for clinical applications. The glutaminase activity of asparaginase

has been implicated in many ALL treatment-associated side effects including immune suppression, pancreatitis, liver damage, and neurotoxicity. A potential strength of glutaminase-deficient asparaginase variants is therefore, the possibility of improved therapeutic index if the modified asparaginase remains active against the cancer cells [29, 30].

In seeking a mutagenesis target that would suppress glutaminase activity but not asparaginase activity, so the molecular structure of the E coli asparaginase active site in complex with aspartic acid investigated in the 3ECA x-ray crystal structure and docking studies were carried out to select D90A substitution as mutant candidate point. The binding energy for WT when docked with glutamine was found to be -7. 43 Kcal/mole, whereas for mutated protein it was found to be -4. 39 Kcal/mole. Binding energy calculation for WT and mutant asparaginase when docked with alanine revealed no significant difference. Based on enzyme activity calculated results, it was revealed that replacement of active site with amino acids other than alanine did not show considerable change in both asparaginase and glutaminase activities. The designed enzyme model with reduced glutaminase side activity was used to develop a variant of enzyme drug through protein engineering by site-directed mutagenesis.

Experimental mutagenesis at residue D90A mutant assessment revealed that replacement of enzyme's active site amino acid Asp90 with alanine decreased the glutaminase catalytic efficiency by 119 percent whereas just 21. 9 % decrease change in Asparaginase kinetic efficiency. So our predictions of mutant behavior correlated well with experimental measurements of asparaginase and glutaminase kinetic parameters.

One of the factors studied in this study is the enzyme pH profile. According to the results, the mutation has no effect on the optimal pH of the mutant in compare with WT enzyme with maximum activity at pH 9. Another point is the bell-shaped in the diagram of the enzyme optimal pH, which is due to the evolution of the pH of the enzyme, gradually resulting in changes in the electrostatic forces as well as in the types of weak links that resulted in the alteration of the enzyme structure and its performance is weakened. Thus, at higher pH and also less than the optimum pH, we see a gradual decrease in the enzyme activity, which results in the formation of a bell-shaped diagram in the enzyme, and both the wild and mutant enzymes have an optimal activity at 40 °C and at temperatures the higher and lower levels of this activity were reduced and did not result in changes in the optimal temperature change in the asparaginase enzyme, which indicates that the enzyme basic structure is not altered by mutation, which has not altered the essential properties of the enzyme. So our predictions of WT and mutant models correlated well with experimental.

Conclusion:

Bioinformatics tools during rational selection and investigation of target amino acid for design and substitution could be deemed as an appealing alternative for conventional experimental mutagenesis methods and would drastically decrease the required time and expenses. The D90A substitution has led to a slight decrease in kinetic efficiency of asparaginase but highly reduction in glutaminase. Also, the mutation does not have an effect on optimal pH, temperature and enzyme activity. So we suggest that could be potential for further drug development research. Nevertheless, preclinical

studies using appropriate in vitro and/or animal models will be critical for determining whether D90A exhibits improved therapeutic index.

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